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SINGLE DIMENSION, MULTIPLE DEVELOPMENT THIN-LAYER CHROMATOGRAPHY OF SUGARS FOR DENSITOMETRIC QUANTIFICATION

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ABSTRACT

Efficacy of certain combinations of thin-layer chromatography media and solvents in qualifying and quantifying sugar mixtures is illustrated. Mixtures were partitioned into subgroups and then resolved into their constituents by selective use of media, solvents, and the technique of multiple development. Acceptance was further based upon applicability for in situ densitometric quantification, such as providing discrete separations, minimum background interferences, and circular to elliptical spot configurations.

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The utility of thin-layer chromatography (TLC) in qualitative and quantitative analyses of the components of sugar mixtures is documented in numerous publications (DeStefanis and Ponte 1968; Hay and others 1963; Jeffrey and others 1969; Ovodov and others 1967; Vomhof and Tucker 1965; Wolfrom and others 1965 and 1966). Thin-layer chromatography has inherent features that make it a desirable tool for quantification of carbohydrates in plant tissues. These features include numerous combinations of chromatographic adsorbents and solvents, convenience of separations of mixture constituents in short development times, and capability of using small quantities of samples.

With TLC techniques, sugars, ranging from simple molecules to polymers, are identified and quantified in many kinds of plant materials. However, selection of appropriate TLC adsorbents and solvents evolves from research needs or sets of special circumstances associated with the sugar constituents being studied and so are derived empirically. Our research needs would be satisfied through use of a densitometer to quantify some common sugars extracted from western white pine tissues. The techniques reported here were those we selected using commercially available compounds and are those we found most applicable to identification and densitometer instrumentation.

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Lack of prior knowledge of the qualitative nature of the sugar mixture in an extract of plant material can result in considerable trial and error in selecting the appropriate TLC system. Time spent in this activity can be minimized by employing the principles used by Gordon and others (1962). TLC adsorbents and solvents are used that first partition sugar mixtures into such general groups as pentoses, hexoses, and disaccharides. Then, the constituents of interest within a group can be resolved from each other with select solvents and multiple developments (Thoma 1963) since the sequence of R_f values for carbohydrates is the same in most solvents, except phenol-water (Isherwood and Jermyn 1951).

Quantitation via in situ densitometry of sugars on TLC media imposes more stringe qualifications for acceptable chromatographic separations than qualitative studies of the same sugar mixtures. The TLC system must provide spot geometries compatible with the densitometer, achieve high resolutions of sample constituents, and must result in minimal background interferences (Chandler and Barton 1955; Goldman and Goodall 1968; Novacek 1972). Each or all of these problem elements can contribute error to the extent that mixtures of soluble sugars of special interest in plant physiology researc cannot be quantitatively analyzed.

EXPERIMENTAL

Developing solvents

The following solvents were used for the separation of monosaccharides, disaccha ides, trisaccharides, and tetrasaccharides. Proportions in parts by volume follow:

A. 1-butanol:acetic acid:water,8:2:3
B. Chloroform:acetic acid:water,50:35:5
C. Isoamyl alcohol:pyridine:water,4:4:1
D. Ethyl acetate:pyridine:water,8:2:1
E. Ethyl alcohol:pyridine:water,8:2:1

Localizing reagent

2 g diphenylamine, 2 ml aniline, 200 ml acetone, and 20 ml phosphoric a (85%).

TLC media

Eastman	#6060, silica gel, 100μ, polyvinyl binder, acetate sheets.
	#6064, cellulose, 160μ, no binder, acetate sheets.
Brinkman	#5765, silica gel, 250μ, unspecified organic binder, glass pla
	#5754, cellulose, 80μ, no binder, glass plate.
Gelman	ITLC-SA, impregnated glass fiber.
	ITLC-SG, impregnated glass fiber.

Activation of TLC sheets

Each 5- by 20-cm acetate sheet coated with silica gel adsorbent was dipped in a 0.1 M monobasic potassium phosphate solution and then dried for 90 minutes at 85 (Lato and others 1968; 1969). The dried sheets were stored over calcium chloride desiccant at room temperature until needed.

Chromatographic procedures.

The sugars were dissolved in distilled water and applied to the chromatograp adsorbents with a microsyringe in 0.1 μl quantities (with intermittent cool dryin until 2 μg of each sugar was accumulated. When 1 μg quantities of each sugar wer used, color intensities for all except glucose were barely visible, whereas incre

in quantities beyond 3.5 to 4.0 µg exceeded the capacity of the TLC media and resulted in such excessive tailing of spots that separations between some sugars were obliterated. The plates were developed in preequilibrated battery jars or ITLC chambers.

Detection of carbohydrate compounds

The developed plates were air dried and dipped into the localizing reagent. Silica gel plates were air dried for 15 minutes and then placed in a 100°C oven for 10 minutes. Cellulose plates were heated for 4-5 minutes at 110°C.

Densitometric procedure

Sheets and plates were cut into strips to fit the densitometer carrier. Scans were performed at 1:1 and 1:3 scan to recorder speeds by using a 300-400 nm light filter and a slit 10 mm by 0.5 mm or 10 mm by 1.0 mm in width or a variable width slit insert.

RESULTS AND DISCUSSION

In our laboratory, we have selected TLC media, developing solvents, and localizing techniques that provide cursory identification of sugar groups within mixtures fabricated from commercial sources. Then, certain developing solvents were used to resolve these groups into their constituents. Criteria for in situ densitometric quantitations such as discrete separations, minimum background interferences, and circular to elliptical spot configurations were met by the systems reported.

TLC Media

ITLC-SA and ITLC-SG sheets were unsatisfactory with any of the solvents tested. These media handled well and accepted the test samples without evident problems, but were unacceptable for quantification of individual sugars because of excessive tailing and absence of resolution between sugars within a group.

Brinkman glass plates also proved to be troublesome for our specific applications since scoring them into inch-wide strips to fit the densitometer carriage resulted in many unusable chromatograms. Samples were easily applied to either cellulose or silica gel adsorbents. However, overdrying as aliquots were applied to both adsorbents resulted in samples staying tenaciously at the origin. This spot, localized at the origin, is easily recognized by its circular configuration and highly delimited border and should not lead to false interpretations.

Silica gel media provided backgrounds that were superior to cellulose adsorbents for measurements of sugar concentrations with in situ densitometric instrumentation (fig. 1). The reaction of the localizing reagent with the cellulose media produces a background that varies from light to dark blue within and between chromatograms. Errors in quantitation resulting from background interferences were large enough to mask differences between sugar concentrations. In contrast, the white background of silica gel adsorbents provided a consistently low integrator baseline and easy visualization of the blue, yellow, and green colors of localized sugars (fig. 1-A).

Development Techniques

Although both cellulose and silica gel adsorbents, in combination with specific solvents, provided in three developments reproducible separations between certain sugars acceptable for quantitation of individual sugars, our impression was that silica gel impregnated with phosphate provided the best resolution. All adsorbent solvent combinations were useful in qualitative applications preliminary to quantitative analysis. In all instances, one development was of marginal value, two developments provided greater insight into the constituents of a sugar mixture, and three developments provided the resolution required for quantification (fig. 1-B). However,

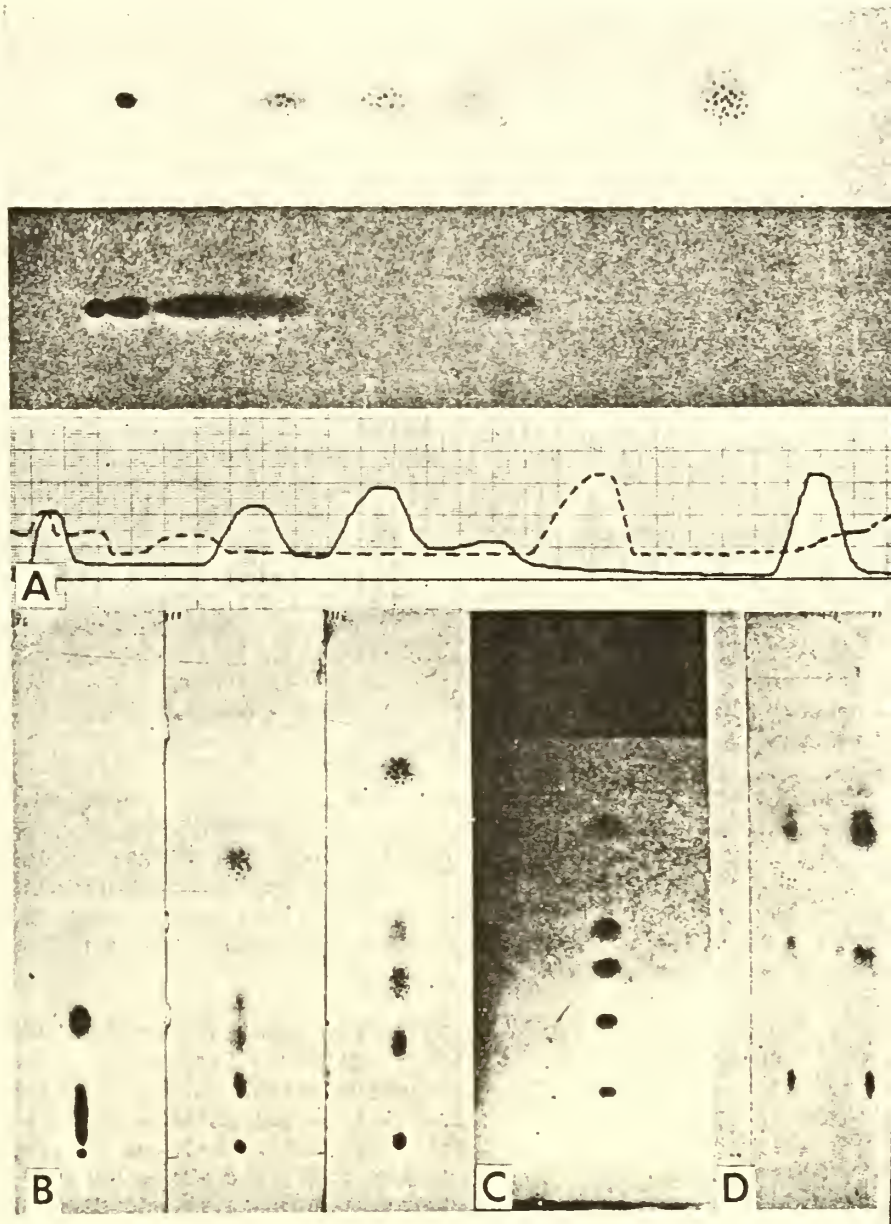

Figure 1.—A. Densitometer traces illustrating differences in light ab silica gel (upper TLC strip, solid trace) and cellulose media (low dash trace). Scan to trace ratio 1:3. B. Three step multidevelop of rhamnose, fructose, glucose, sucrose, and raffinose. Solvent D C. Preliminary qualification of a mixture of sugars with solvent A three developments. Black mask used to illustrate solvent front. trisaccharides and tetrasaccharides from the origin into the lower silica gel chromatogram and resolution between raffinose and stach with three developments in solvent C.

solvent A (1-butanol:acetic acid:water) in combination with either silica gel or cellulose was the only exception to production of circular or slightly elliptical spots. This feature distracts from its usefulness in quantitative, but not in qualitative, analyses of sugar mixtures.

Solvents

Mixtures of sugars were first partitioned into groups and then select groups partitioned into the constituents by means of certain combinations of solvent, media, and multiple ascending developments (table 1). Solvents containing ethyl acetate had an appreciably shorter time for each development than solvents containing 1-butanol or chloroform. Of all solvent and adsorbent combinations tried, solvent A in combination with cellulose gave the best spread of a mixture of sugar groups (fig. 1-C). In conjunction with movement of all compounds from the origin, the separation of sugar groups utilized nearly the entire length of the solvent migration. Pentoses occupied an area of the chromatograms nearest the solvent front. Hexoses and disaccharides were found midway on the chromatograms, and high molecular weight trisaccharides and tetrasaccharides near the origin. The combinations of solvent C and cellulose or silica gel, or solvent D and silica gel were almost equivalent to the above system in separating capacity. However, fructose, glucose, and sucrose are very close together; consequently, the possibility of other sugars being in this area would be overlooked. The benefits of solvent C and silica gel are the separation of raffinose and stachyose and their movement away from the origin allowing quantification and permitting identification of the number of sugars in this area (fig. 1-D).

Table 1.--*Media and solvent combinations used to resolve certain sugar mixtures. Position in table relative to other groups is similar to area occupied on chromatogram*

Cellulose TLC media		Silica gel TLC media	
Sugars	Solvents	Sugars	Solvents
Rhamnose Ribose Xylose Fructose	Ethyl acetate:pyridine:water(8:2:1)	Rhamnose Ribose Xylose Fructose	Ethyl acetate:pyridine:water(8:2:1)
Arabinose- fructose Sorbose Mannose Glucose	Chloroform:acetic acid:water(50:35:5)		
Sorbose- fructose Glucose Galactose Sucrose	Ethyl acetate:pyridine:water(8:2:1)		
		Glucose Galactose Lactose- melibiose Raffinose	Chloroform:acetic acid:water(50:35:5)
Melezitose Raffinose Cellobiose	Chloroform:acetic acid:water(50:35:5)	Cellobiose Melezitose Raffinose	1-butanol:acetic acid:water(8:2:3)

Two solvents, A and B, were satisfactory in carrying disaccharides, trisaccharides and tetrasaccharides from the origin to the center area of chromatograms, provided the proper TLC media were used. For example, mixtures of four disaccharides were separated into sucrose, maltose, and lactose-melibiose spots with solvent B and silica gel media, but no separations occurred when cellulose media were used. The disaccharide and trisaccharide mixture, cellobiose, melezitose, and raffinose, separated into three spots in the solvent A-silica gel combination, but extended into a long, indecipherable spot when solvent B was used.

Partitioning of simple sugars within the pentose and a methylated derivative, rhamnose, of the hexose group was accomplished with ethyl acetate, pyridine, and water (solvent D) on cellulose media. Within this group of sugars, rhamnose, ribose, xylose, and fructose were satisfactorily separated from each other, with the entire group occupying more than the upper half of the chromatogram. Fructose, glucose, sucrose, and raffinose separated well, but with not enough distance between sugars to allow identification of other hexoses and disaccharides. When using the same solvent on silica gel, a greater separation between rhamnose and ribose and between xylose and fructose occurred, but ribose and xylose were contiguous.

Chloroform, acetic acid, and water (solvent B) in combination with cellulose provided satisfactory separations between sugars of a mixture of hexoses and disaccharides. Glucose, mannose, and sorbose were separated from each other and from a spot containing arabinose and fructose. Separations between constituents having positions lower than glucose on the chromatograms were found to be satisfactory in the solvent D-cellulose media combination. This method was used to separate glucose, sucrose, galactose, and raffinose from each other, at the expense of sorbose and fructose, which occupied a spot above glucose.

SUMMARY

Because of the diversity of sugar compounds, it is unlikely that a single solvent system will completely resolve a mixture of them into discrete spots on a chromatogram acceptable for densitometric quantitation. However, solvents and TLC media combinations can be used to fractionate a mixture into groups within which the sugars have some common characteristics. Solvents and TLC media effective in influencing the migration of the group constituents can then be used to resolve them and also to provide symmetrical circular or elliptical spots.

We found solvent A (1-butanol:acetic acid:water) in combination with cellulose to be a satisfactory system for the partitioning of a mixture of a pentose, hexose, disaccharide, trisaccharide, and tetrasaccharide over the distance traveled by the solvent. All solvent and media combinations provided the same sequential order of these groups, but differed in influencing the spread between and within groups. In applying our findings to quantitative analysis of pine tissues, the ethyl acetate, pyridine, and water solvent with silica gel was preferred to separate three sugars within the pentose and methylated hexose derivative group. Chloroform, acetic acid, and water solvent performed best in separating for quantification the constituent pine hexoses and disaccharides or disaccharide, trisaccharide, and tetrasaccharide mixtures.

LITERATURE CITED

Chandler, C., and L. V. Barton.
1955. Morphological and physiological studies of diploid and tetraploid *Plantago ovata* Forsk. Contrib. Boyce Thompson Inst. 18:193-214.

DeStefanis, V. A., and J. G. Ponte, Jr.
1968. Separation of sugars by thin-layer chromatography. J. Chromatogr. 34: 116-120.

Goldman, J., and R. R. Goodall.
1968. Quantitative analysis on thin layer chromatograms: a theory for light absorption methods with an experimental verification. J. Chromatogr. 32:24-42.

Gordon, H. T., W. W. Thornburg, and L. N. Werum.
1962. Rapid paper chromatographic fractionation of complex mixtures of water-soluble substances. J. Chromatogr. 9:44-59.

Hay, G. W., B. A. Lewis, and F. Smith.
1963. Thin-film chromatography in the study of carbohydrates. J. Chromatogr. 11:479-486.

Isherwood, F. A., and M. A. Jermyn.
1951. Relationship between structure of the simple sugars and their behaviour on the paper chromatogram. Biochem. J. 48:515-524.

Jeffrey, D. C., J. Arditti, and R. Ernst.
1969. Determination of di-, tri-, and tetrasaccharides in mixtures with their component moieties by thin layer chromatography. J. Chromatogr. 41: 475-480.

Lato, M., B. Brunelli, G. Ciuffini, and T. Mezzetti.
1968. Bimensional thin-layer chromatography of carbohydrates on silica gel impregnated with boric acid. J. Chromatogr. 34:26-34.

Lato, M., B. Brunelli, G. Ciuffini, and T. Mezzetti.
1969. Thin-layer chromatography of carbohydrates on silica gel impregnated with sodium acetate, monosodium phosphate and disodium phosphate. J. Chromatogr. 39:407-417.

Novacek, V. M.
1972. Errors in quantitative analysis of thin-layer chromatograms. Am. Lab. 5:85-91.

Ovodov, Yu. S., E. V. Evtushenko, V. E. Vaskousky, R. G. Ovodova, and T. F. Solovieva.
1967. Thin-layer chromatography of carbohydrates. J. Chromatogr. 26:111-115.

Thoma, J. A.
1963. Application and theory of unidimensional multiple chromatography. Anal. Chem. 35:214-224.

Vomhof, D. W., and T. C. Tucker.
1965. The separation of simple sugars by cellulose thin-layer chromatography. J. Chromatogr. 17:300-306.

Wolfrom, M. L., R. M. deLederkremer, and G. Schwab.
1966. Quantitative thin-layer chromatography of sugars on microcrystalline cellulose. J. Chromatogr. 22:474-476.

Wolfrom, M. L., D. L. Patin, and R. M. deLederkremer.
1965. Thin-layer chromatography on microcrystalline cellulose. J. Chromatogr. 17:488-494.